

Phytol Metabolites Are Circulating Dietary Factors that Activate the Nuclear Receptor RXR

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of material in active fractions pointed to the saturated diterpenoid phytanic acid, which induced RXR-dependent transcription a ¹⁰⁰ fold higher than phytyl ester. This compound was 200 times more potent than phytanic acid, 9cRA was 100 times more potent than phytyl ester. The activity of extract and cannot be present at a concentration of 100 nM. The compound was synthesized and stimulated RXR with a

potencies match their micromolar circulating concentrations. Given their exclusive dietary origin, these chlorophyll metabolites may represent essential nutrients that coordinate cellular metabolism through RXR-dependent signaling pathways.

INTRODUCTION

Nuclear receptors are transcription factors that regulate gene expression in response to lipophilic ligands such as steroid hormones (Yamamoto, 1985). Ligand binding increases the receptor affinity for hormone-responsive DNA elements (HREs) near target genes that promote specific transcriptional control (Glass, 1994). A large family of receptors coordinates cell physiology through these hormone-regulated gene

networks (Evans, 1988). The kindred includes struc-

Activators for orphan receptors have been found by testing compounds in cells transfected with the corre-

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¹ Abbreviations used: ATRA, *all-trans* retinoic acid; FBS, fetal bovine serum; 9cRA, *9-cis* retinoic acid; RAR, retinoic acid receptors.

sponding receptor and HRE-linked reporter genes (Giguere *et al.*, 1986; Green and Chambon, 1987). Aldosterone, retinoic acid, and ecdysone are some of the ligands matched with receptors via these "cis-trans" assays (Arriza *et al.*, 1987; Giguere *et al.*, 1987; Petkovich *et al.*, 1987; Koelle *et al.*, 1991). The nanomolar affinities of these ligands contrast with the micromolar amounts of fatty acids or prostaglandin J₂ required to activate PPAR α and PPAR γ , respectively (Gottlicher *et al.*, 1992; Keller *et al.*, 1993; Forman *et al.*, 1995b; Kliewer *et al.*, 1995). Similarly, metabolites of farnesyl pyrophosphate (farnesoids) are needed at micromolar levels to induce FXR (Forman *et al.*, 1995). Farnesoids and farnesoids have been identified as biological effectors for PPAR γ (Keller *et al.*, 1993; Weinberger, 1996). Ligands related by PPAR are linked to fatty acids have been detected in PPAR-inducing chromatographic fractions from human plasma (Banner *et al.*, 1993), direct interactions of fatty acids with PPARs have not yet been demonstrated.

RXR is a unique member of this orphan receptor family that facilitates many signaling pathways by heterodimerizing with receptors activated by thyroid hormones, retinoids, vitamin D, fatty acids, and farnesoids (Manglesdorf and Evans, 1995). RXR partners also include the orphan receptors COUP (Kliewer *et al.*, 1992), NGF1b/nurr1 (Forman *et al.*, 1995c; Perlmann and Jansson, 1995), and UR/LXR subfamily members (Song *et al.*, 1994; Teboul *et al.*, 1995; Willy *et al.*, 1995). The variety of these interactions suggests that RXR performs a key regulatory role in cell physiology.

Surveys of chemical compounds revealed *all-trans*-retinoic acid (ATRA)¹ as an RXR inducer (Manglesdorf *et al.*, 1990). However, ATRA did not bind RXR with high affinity, supraphysiological levels were required for activity, and receptors for retinoic acid (RAR) had already been identified (Giguere *et al.*, 1987; Petkovich *et al.*, 1987). Thus, it was proposed that ATRA might be metabolized to a more active form (Manglesdorf *et al.*, 1990). Indeed, ATRA isomerizes to 9-*cis*-retinoic acid (9cRA), which activates RXR with a greater potency (Heyman *et al.*, 1992; Levin *et al.*, 1992), but activation of RXR and RAR by 9cRA limits its physiological specificity (Allegretto *et al.*, 1993). Identification of RXR-specific synthetic "retinoids" and methoprene acid (Lehmann *et al.*, 1992; Boehm *et al.*, 1994, 1995; Harmon *et al.*, 1995), coupled with an inability to detect 9cRA in rat serum (Kojima *et al.*, 1994), may argue for the existence of other endogenous RXR-selective terpenoids.

An important question emerging from receptor ligand searches is the following: How are the pharmacological activators to be distinguished? Activity is one measure, but activity with its abundance in the cell is critical. That is, the intra-

MATERIALS AND METHODS

Reagents

Fatty acids and other chemicals for enzyme assays were purchased from Sigma Chemical (St. Louis, MO). Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD).

Cell Culture and Transfections

plasmid DNAs were added per 10^5 transfected CV-1 cells. For the TK-(CRBP_{II})-LUC plate, 300 ng TK-(CRBP_{II})-LUC, 500 ng CMX- β -gal, and CMX-hRXR α were added per 10^5 transfected CV-1 cells.

Enzyme Assays

tivity (20 μ l; Seed and Sheen, 1988) or β -galactosidase activity (2 μ l; Herbolme *et al.*, 1984). Luciferase activity was measured as described in Berger *et al.* (1992).

Bovine Serum Extraction

FBS (Life Technologies) or serum from bovine blood (freely grazing steer raised on silage at North Carolina State University School of Veterinary Medicine) were extracted with chloroform and methanol solvents (Bligh and Dyer, 1959). Briefly, 10 ml of serum was mixed with 37.5 ml of chloroform and methanol (2:1) and vigorously shaken for 15 min. The mixture was centrifuged at $2000 \times g$ for 20 min. To the supernatant was added 12.5 ml each of water and chloroform to separate the phases. The mixture was centrifuged at $9000 \times g$ for 15 min, and the chloroform phase was collected. Alternatively, serum was saponified (2 M KOH at 70°C for 30 min) and twice extracted with diethyl ether. Next, the aqueous solution was acidified with concentrated HCl and then extracted with ether again. Chloroform, methanol, or ether was removed by rotary evaporation under vacuum (Buchi Rotavapor R-124 or Speed Vac SC210A; Savant, Farmingdale, NY).

High Performance Liquid Chromatography

Pure chemical standards or bovine serum extracts were resuspended in 80% methanol and injected into a 1 ml Rheodyne sample loop connected to a Beckman System Gold high-performance liquid chromatography unit (HPLC). The LC system consisted of an RP18 guard column (15 \times 3.2 mm, RP18; Alltech, Deerfield, IL) linked to a separation column (4.6 \times 25 cm, Econosphere C18, 5 μ particle size; Alltech) and a Gilson FC 203B fraction collector (Middleton, WI). UV absorbance was monitored with a Beckman diode array detector module 168. The sample was eluted with an 80% methanol/20% 10 mM ammonium acetate mobile phase for 5 min, after which a linear gradient (80–100% methanol, 20 min) was applied and held at 100% methanol for 10 min. Fractions were collected, dried, and dissolved in DMEM/F12 containing 5% dextran-coated charcoal-adsorbed FBS for measurement of CAT activity in the cis-trans assay.

Silica Gel Chromatography

Pure phytanic acid or a chloroform extract of bovine serum was loaded on a silica gel column (4 cm wide \times 10 cm height) and eluted with 20% ethyl acetate/80% hexane. In all, 8-ml fractions were collected in 13 \times 100-mm glass test tubes, dried by rotary evaporation, resuspended in media containing charcoal-adsorbed FBS, and tested in the cis-trans assay as described.

Mass Spectroscopy

Gas chromatography/mass spectrometry (GC/MS). The trimethylsilyl (TMS) derivative of serum fraction 23 and the phytanic acid standard were prepared by reacting 5 μ l of each sample with 10 μ l of *N,O*-bis(trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA). Reaction mixtures were heated at 70°C for 15 min. An aliquot

(0.5 μ l) of the reaction mixture was injected onto a Quadrex methylphenyl 5 capillary GC column (30 \times 0.25 mm ID, 0.25 mm film) in

Fast atom bombardment. A VG ZAB-4F magnetic sector instrument was used to obtain fast atom bombardment (FAB) data at an accelerating voltage of 8 kV. An Ion Tech atom gun and xenon atoms were used to bombard the sample. The samples were introduced into the mass spectrometer via a coaxial continuous-flow FAB interface. This interface uses a coaxial arrangement of fused silica capillaries to independently deliver the FAB matrix (glycerol) and the analytes. The instrument was scanned from 1000 to 100 daltons at 5 s/decade to acquire the full-scan negative ion data.

Electrospray/ionization MS. Measurements were made on a Fisons-VG Quattro BQ triple-quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ion source operating at atmospheric pressure. The HPLC fractions containing biologically active material and phytanic acid were reconstituted in acetonitrile and mixed with equal volumes of the LC mobile phase (80% acetonitrile/20% water containing 1% ammonium hydroxide). Samples were introduced by loop injection into the mobile phase at a flow rate of 8 μ l/min, and spectra were acquired in the negative ion continuum-mode scan rate. The mass scale was calibrated with polyethylene glycol with an average molecular weight of 400 atomic mass units (amu). Theoretical isotope distributions were computed with Fisons Instruments Opus software.

Synthesis of Phytanic Acid

Phytanic acid was prepared from phytol (Sigma) by adapting a two-step MnO₂ oxidation procedure (Corey *et al.*, 1968). Phytol was oxidized to phytal by using activated MnO₂ (Aldrich Chemical,

ene group is 2.07 ppm. For the cis isomer, the methyl group is relatively shielded by the carbonyl group (1.84 ppm), and the methylene group is relatively deshielded (2.56 ppm).

Hormone Binding

[³H]-ATRA or [³H]-9cRA binding to baculovirus-expressed RAR(α , β , γ) or RXR(α , β , γ) polypeptides was measured as described previously (Allegretto *et al.*, 1993). Receptor genes expressing these recombinant proteins were all of human origin except RXR β and RXR γ , which were derived from the mouse. The assay buffer consisted of 8% glycerol, 120 mM KCl, 8 mM Tris-HCl, 5 mM CHAPS, 4 mM dithiothreitol, and 0.24 mM phenylmethylsulfonyl fluoride, final pH 7.4 (room temperature). The final volume for binding assays was 250 μ l, which contained 10–40 μ g of protein extract plus 5 nM of [³H]-ATRA for RARs or 10 nM [³H]-9cRA for RXRs, plus varying concentrations of competing ligands. Incubations were performed at 4°C until equilibrium was achieved. Nonspecific binding is defined as that binding remaining in the presence of 1 μ M of the appropriate unlabeled retinoid isomer. At the end of the incubation,

50 μ l of 6.25% hydroxylapatite was added in the appropriate wash buffer (100 mM KCl, 10 mM Tris-HCl, and either 5 mM CHAPS [RXRs] or 0.5% Triton X-100 [RARs]) to bind the receptor-ligand complexes. Mixtures were vortexed and incubated at room temperature for 30 min and centrifuged, and the supernatants were removed. Hydroxylapatite beads were washed two more times with the same buffer. Receptor-ligand complexes were determined by scintillation counting of the pellets. After correcting for nonspecific binding, specific binding was determined. The IC_{50} value is the concentration of competing ligand required to decrease specific binding by 50%, which is determined graphically from a computer-based log-logit plot of the data (Cheng and Prusoff, 1973).

RESULTS

RXR Effector Activity from Bovine Serum

We initially attempted to identify activators from bovine serum (Shih *et al.*, 1991) for an orphan receptor called OR6, which binds to an AGGTCA direct repeat HRE separated by 4 bp (DR4), but only in the presence of RXR (Umesono *et al.*, 1991). CHO cells were transfected with a DR4-linked CAT reporter plasmid DNA along with an OR6 expression vector, and CAT activity was measured. A lipid extract of FBS was added (Bligh and Dyer, 1959), but this had no effect on CAT activity (our unpublished observations). Although the extract stimulated activity eightfold when RXR was added, RXR alone showed a similar effect (our unpublished observations). These results suggested that the bovine serum activator was mediating its effects through RXR.

Therefore, the serum effector was compared with 9cRA, a previously described RXR effector from liver (Heyman *et al.*, 1992). The chloroform extract of serum was separated by reverse-phase HPLC, and the eluted fractions were tested for RXR effector activity. Unexpectedly, the RXR activator had a retention time (R_t) between 19 and 22 min (Figure 1), which did not coincide with the elution profile for a 9cRA standard (R_t = 7 min). Because 9cRA is chemically similar to ATRA, we added a tracer amount of [3 H]-ATRA (1 nM) to a serum sample to ask whether retinoic acid could be extracted by this method. Nearly all of the radioactivity (83%) was found in the chloroform fraction, thus supporting the utility of the Bligh and Dyer method for extracting retinoids (our unpublished observations).

RXR Effector Activity Is Distinct from 9cRA

It is possible that the serum may have been contaminated by 9cRA. To rule out this possibility, we used the Bligh and Dyer method, which is designed to extract retinoids but not 9cRA, to isolate retinoids was

used as potential sources of retinoids that are sometimes given to cattle. The serum from a freely grazing

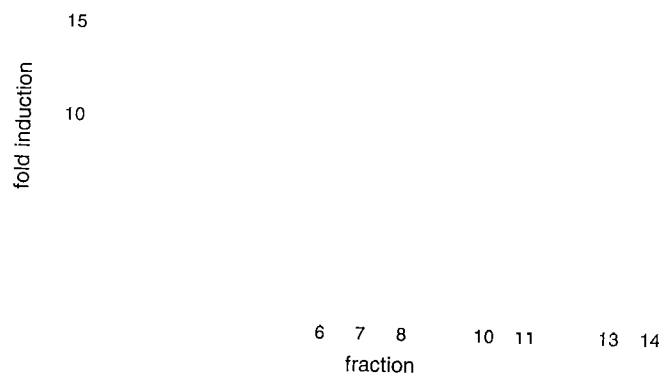


Figure 1. Identification of an RXR effector activity from bovine serum. RXR effector activity profile from chloroform extract of FBS fractionated by reverse-phase HPLC. The chloroform fraction from a Bligh and Dyer extract of 20 ml of FBS was separated by reverse-phase HPLC methods, as described in MATERIALS AND METHODS. Two-minute fractions were pooled and tested for RXR effector activity with the cotransfection assay. CHO cells transfected with 1.25 μ g of pCMX-mouse RXR α , 0.25 μ g of pCMX-mouse CAT, and 1.25 μ g of pCH111 plasmid (Yao *et al.*, 1991) were transfected with the cotransfection assay. A 9cRA standard had a retention time of 7 min via this method. The experiment was performed three times with similar results. Note that the coefficient of variation for CAT activity measurements is typically <15%.

ether extracted, and then the aqueous phase was acidified and extracted with ether again. [3 H]-ATRA in a parallel sample was quantitatively extracted by ether (95%) from the acidified aqueous solution, marking this as another effective means for retinoid isolation. In contrast, RXR-inducible CAT activity was found only in the ether extract (our unpublished observations). The RXR effector activity was identified by the HPLC condition used in Figure 1, and the 1-min fraction was tested for RXR effector activity. An RXR-specific activator (R_t = 23–24 min, Figure 2A) was identified that eluted later than ATRA or 9cRA (R_t = 8.8 and 7.5 min, respectively). Therefore, both saponified and nonsaponified serum extracts contained an RXR activator with chromatographic properties distinct from 9cRA.

It was conceivable that retinoids were destroyed by this rigorous extraction method. Therefore, DNAs for the human retinoic acid receptor (RAR α) and β RARE-CAT reporter were transfected into cells to permit detection of the RAR activators ATRA and retinol (Giguere *et al.*, 1987; Sucov *et al.*, 1990). Activities coincident with 9cRA, ATRA (R_t = 7.5 and 8.8 min), and retinol (R_t = 20 min) were confined to the acidified extract (Figure 2B); none

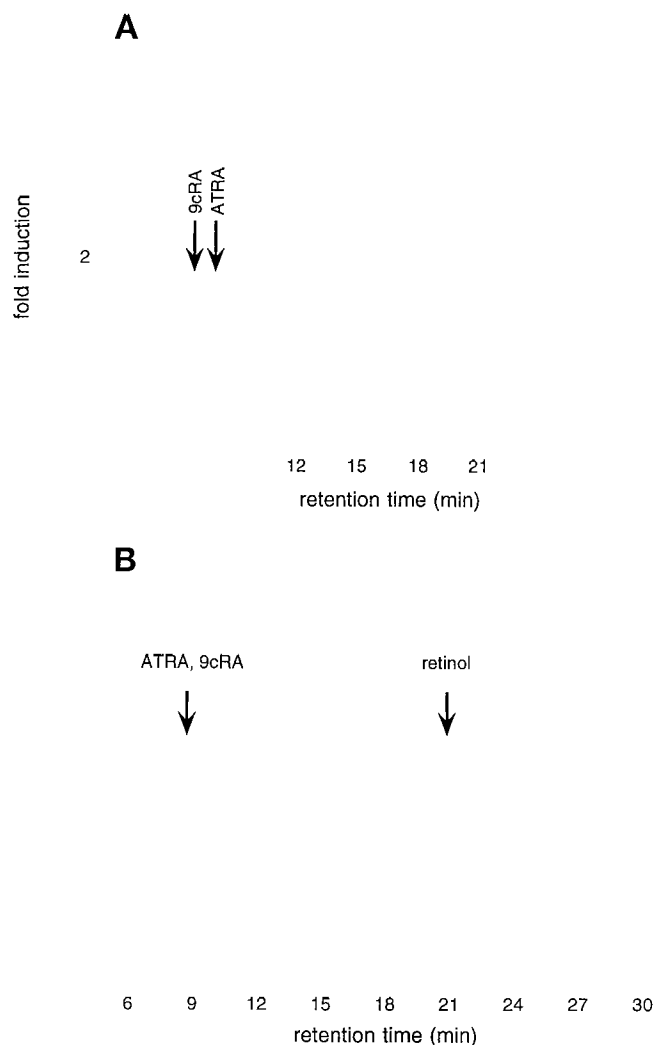


Figure 2. RXR and RAR activators in bovine serum can be extracted by saponification and ether extraction. Bovine serum (10 ml) was saponified (2 M KOH, heated at 70°C for 30 min) and extracted with diethyl ether. The aqueous phase was acidified, and ether was extracted again. Basic and acidic ether extracts were dried and fractionated separately by reverse-phase HPLC, as described in MATERIALS AND METHODS. (A) Separation of RXR activators from bovine serum by reverse-phase HPLC. One-minute fractions were collected and dried, and a portion (5%) was taken up in a medium containing 5% charcoal-adsorbed FBS for testing by *cis-trans* assay, as described in Figure 1. (B) Characterization of RAR activators from bovine serum by reverse-phase HPLC. Three-minute fractions were tested by cotransfecting an SV-(β RARE)₂-CAT reporter plasmid and a plasmid DNA expressing the human RAR α receptor into CHO cells, essentially as described in Figure 1. Symbols: shaded bars, acidic extract; closed bars, basic extract. Fold induction values are relative to control samples containing methanol vehicle. A control sample in B containing 200 nM ATRA showed a 4.9-fold induction by comparison.

was found in the ether extract of the basic solution in which the RXR effector activity was observed. In addition, a broad range of activity more polar ($R_t < 20$ min) than retinol was seen. This material may correspond to hydroxylated retinol metabolites,

such as 4-oxo-retinol, the acid derivative of which was shown to activate RAR (Pijnappel *et al.*, 1993). Nevertheless, although peaks of activity cannot be assigned, it is clear that RAR and RXR activators have distinct pH-dependent partitioning characteristics in ether. Moreover, the functional integrity of RAR activators is maintained during extraction. By inference, 9cRA should have been found in the acidic fraction, but no corresponding RXR effector activity was detected here (our unpublished observations). These results suggest that the bovine serum activator is distinct from 9cRA, but they do not exclude the possibility that 9cRA may still be an intracellular signal in the liver or kidney, where it was originally described (Heyman *et al.*, 1992).

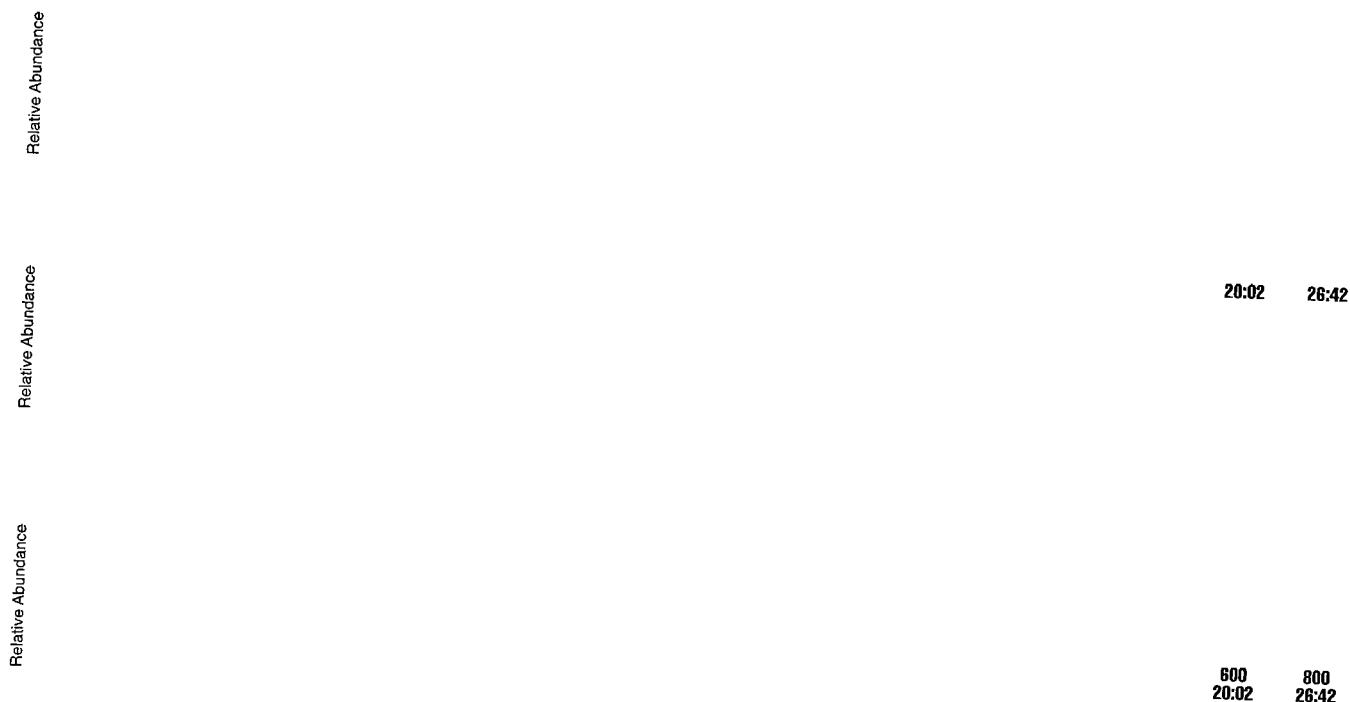
Fatty Acids Copurify with RXR Effector Activity

To characterize the molecular structure of the RXR activator, the active fraction of the basic ether extract ($R_t = 23$ min) and two adjacent inactive ones ($R_t = 22$ and 25 min) were analyzed by various mass spectrometric techniques. Negative ion electrospray spectra, obtained by flow-injection analyses of these fractions, contained ions of m/z 283 and 311 (Figure 3A). The abundance of the m/z 311 ion corresponded to the RXR activities in these fractions (Figure 2A), whereas the abundance of the m/z 283 ion did not follow the RXR activities. Relative isotopic abundance measurements for these negative ions predicted the molecular formulas $C_{18}H_{36}O_2$ and $C_{20}H_{40}O_2$ for the molecular weight 284 and 312 Da components, which are consistent with the elemental compositions of stearic acid and phytanic acid, respectively (our unpublished observations). The same two prominent (M-H)⁻ ions, m/z 283 and 311, were also observed by negative-ion fast atom bombardment mass spectrometry (our unpublished observations).

GC/MS analysis of the TMS-derivatized saponified sample showed a peak corresponding in mass to the (M-CH₃)⁺ fragment ion (m/z 369) of the TMS derivative of phytanic acid, as well as a low-abundance peak corresponding to the molecular ion (m/z 384). The full-scale mass spectrum and the retention time of this component were in agreement with those of the TMS derivative of authentic phytanic acid (Figure 3B), run under identical conditions. Cochromatography of the sample and the phytanic acid standard gave a single peak in the reconstructed ion chromatogram for m/z 369, as well as for other characteristic ions.

Phytanic Acid Is the Serum RXR Activator

A single chromatographic step was deemed unlikely to have separated the RXR activators from other serum components. Nonetheless, the above results prompted us to examine a collection of fatty acids for RXR activation. Although linoleic, oleic, stearic, farne-



idonic acids (40 μ M) were

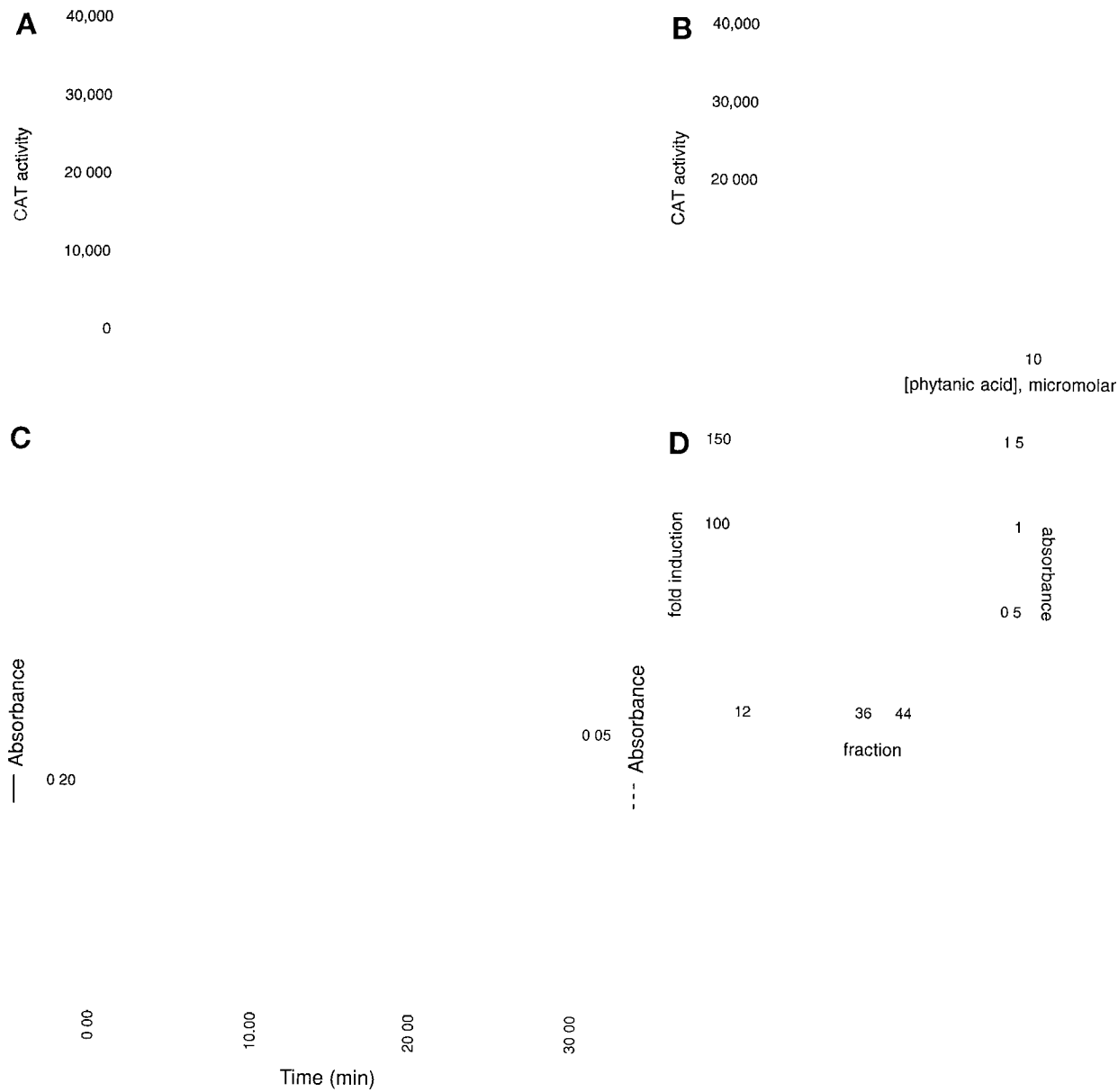
tanic acid standard coeluted with the serum RXR activator when separated by silica gel chromatography with 20% ethyl acetate in hexane as the developing solvent (Figure 4D). Together these results suggest that the RXR activator in serum corresponds to phytanic acid.

Detection of Phytanic Acid

The DR4-CAT reporter plasmid was originally selected for isolating RXR activators from bovine serum in favor of CRBP-II-CAT because of its more stable expression in C2C12 cells (Figure 4A). Despite having not been previously used as a reporter element, and thus

parately inserted in the

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ode array detector module 168. One nanomole of 9cRA was separately
e that the delay time between absorbance measurement and fraction

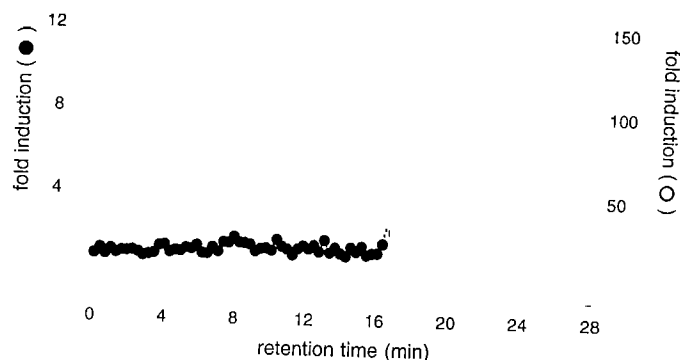
herpes simplex virus thymidine kinase promoter that was linked to the firefly luciferase gene (Forman *et al.*, 1995c). These reporter plasmids were independently cotransfected into CV-1 cells with CMX-GAL4-RXR (Forman *et al.*, 1995c), a chimeric receptor fusing the GAL4 DNA-binding domain to the human RXR α ligand-binding domain, or with CMX-human RXR α (Yao *et al.*, 1993) as the respective receptor plasmids.

A chloroform extract of FBS (Bligh and Dyer, 1959) was separated by reverse-phase HPLC as described in Figure 2B, but this time the eluate was collected in 0.3 min fractions to afford greater analytical resolution. The material was divided in half, each was added to the two sets of CV-1 cells cotransfected as described above, and normalized luciferase activities were measured. The superimposable profiles contained two peaks of activity (19.0 and 21.6 min; Figure 5) corresponding to the absorbance profiles for phytenic acid and phytanic acid, respectively ($R_t = 18.2$ and 20.8 min for this particular column). The amounts of serum extract used for these assays were ~ 10 -fold greater than those used earlier (Figure 2, A and B). Thus, the cytotoxicity shown in two adjacent fractions ($R_t \sim 21$ min) may have been due to increased amounts of phytenic acid now became detectable (Figure 2A), a peak coincident with the results previously obtained with the DR4-CAT reporter plasmid to define both phytenic acid and phytanic acid in bovine serum extracts.

Phytol Metabolites Bind and Activate RXR

Metabolites derived from the phytol (Figure 6A) were compared for stimulation of RXR, using the DR4-CAT reporter plasmid. Synthetic phytol of 40% cis and 60% trans isomers, was tested along with phytenic acid, pristanic acid, and 9cRA. The dose responses for RXR activation by these acids were similar, having EC₅₀s in the same range as pristanic acid, a phytol-related with a lower potency. By comparison, 9cRA induced ~ 200 -fold lower than phytenic acid or phytanic acid. Tests of phytenic acid at 32 μ M induced RXR effector activity 4.5-fold, which paralleled that of phytanic acid, whereas the cis isomer was nearly inactive (our

phytenic acid with RXR was 3 H]-9cRA bound to baculovirus-expressed proteins with unlabeled



JAS_C)₄-LUC reporter and GAL4-CAT reporter plasmid DNAs by liposome-mediated transfection. **MATERIALS AND METHODS.** Fractions of the fractionated extracts were used to assay luciferase activities as described (Berger *et al.*, 1993). Measurements were performed, which typically exhibit 10% variations in this assay. Fold induction is expressed as the relative luciferase activity in the presence of fractionated extracts as compared with untreated cells. The cytotoxicity in two fractions at 21 min is denoted by zero inductions. Duplicate control wells to which the FXR activator JH III (40 μ M) was added showed onefold inductions; those to which the RXR-specific ligand LG69 (100 nM; Boehm *et al.*, 1995) was added produced 25-fold and 110-fold inductions by using CRBP-II-CAT or GAL4-CAT reporters, respectively. Elution positions for phytenic acid and phytanic acid (corresponding to absorbance peaks measured at 220 nm) were $R_t = 18.2$ and 20.8 min, respectively, and are denoted by arrows.

DISCUSSION

Refsum's Disease

The diterpenoid structure of phytanic acid (Sonneveld *et al.*, 1962; Lough, 1964) suggested that it might be synthesized from mevalonate, but neither endogenous biosynthetic routes nor intestinal microbes contribute to circulating pools in mammals (Steinberg, 1965, 1967). Phytol metabolites in animal tissues are exclusively derived from the phytyl side chain of chlorophyll. Phytanic acid may be elevated 50-fold and constitute >20% of the fatty acids in patients with Refsum's disease, an inherited metabolic disorder characterized by an α -hydroxylase gene defect that prevents phytanic acid conversion to pristanic acid (Figure 5A; Steinberg, 1983). The neuropathological signs in these patients may be caused by demyelination induced by α -oxidation of phytanic acid in nerve cells that maintain a preference for long-chain

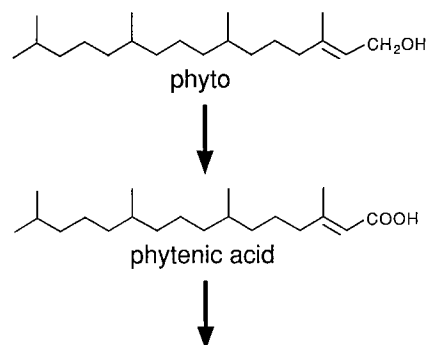
Because these diets raised phytanic acid levels to 30% of total fatty acids just as in Refsum's disease, targeted disruption of the α -hydroxylase gene may provide a better approach for understanding the pathological effects.

Phytol Metabolites as Transcriptional Signals

Phytanic acid and phytenic acid levels in normal human serum are 6 μ M and 2 μ M, respectively (Avignan, 1966). Like other fatty acids, 70% of the phytanic acid probably exists as triacylglycerol or phospholipid esters that are rapidly oxidized and that vary with dietary conditions (Mohrhauer and Holman, 1963; Mize *et al.*, 1966, 1969). Although the estimated free phytanic acid (2 μ M) is only at the threshold for RXR stimulation (Figure 4B), equipotent phytenic acid may also contribute to the RXR effector pool (Mize *et al.*, 1966). In addition, the charcoal-treated serum used in this bioassay may have adsorbed some of the added phytanic acid, thereby reducing its effective concentration. Phytol is unlikely to be an RXR effector because at 50 μ M it neither bound nor activated RXR,

ria *et al.*, 1982).

The EC₅₀ values for RXR activation by phytol metabolites were estimated assuming that the dose-response maxima were reached at 64 μ M (Figure 3A). These nonsaturating dose-response curves are



H

B

CAT activity

10⁻⁷ 10⁻⁶ 10⁻⁵ 10⁻⁴
[activator], M

Figure 6. Various chlorophyll metabolites activate RXR. (A) Metabolic pathway from chlorophyll to pristanic acid. First, the phytol ester is hydrolyzed, which is followed by oxidation to phytenic acid. Phytenic acid is then hydrogenated to phytanic acid and α -hydroxylation; oxidation leads to pristanic acid. Pristanic acid is finally metabolized by fatty acid β -oxidation pathways. The trans isomers of phytol and phytenic acid are illustrated here. (B) RXR effector activity induced by phytol metabolites and 9cRA. Increasing amounts of 9cRA, phytanic acid, phytenic acid (40% cis/60% trans isomer mixture), and pristanic acid were added to cells transfected with the RXR-specific CRBPII-CAT reporter plasmid and mouse RXR α . CAT activity was measured from duplicate wells in an assay configured similarly to that described in Figure 1. Average values for CAT activity from duplicate transfected plates are plotted against increasing activator concentrations. Symbols: circles, 9cRA; diamonds, phytenic acid; squares, phytanic acid; triangles, pristanic acid.

Table 1. Competition of phytol and metabolites for [³H]-ATRA and [³H]-9cRA binding to RXRs

Compound	K _i					
	RAR α	RAR β	RAR γ	RXR α	RXR β	RXR γ
ATRA ^a	18.2 \pm 2.1	17.3 \pm 1.8	14.6 \pm 1.8			
9- <i>cis</i> RA ^a				10.2 \pm 1.5	22.1 \pm 2.3	19.8 \pm 0.6
Phytol ^b	> 100	70 \pm 30	> 100	67.2 \pm 32.8	41.9 \pm 0.2	47.1 \pm 12.6
Pristanic acid ^b	> 100	74.8 \pm 25.3	88 \pm 12	15.1 \pm 8.6	13.3 \pm 3.3	25.6 \pm 17.2
Phytanic acid ^b	> 100	> 100	> 100	4.4 \pm 0.7	4.1 \pm 0.2	3.6 \pm 0.7
Phytenic acid ^b	> 100	> 100	> 100	2.3 \pm 0.4	3.7 \pm 1.1	2.4 \pm 0.4

^a Values are in nM and represent the mean \pm SEM of two determinations.

^b Values are in μ M and represent the mean \pm SEM of two determinations, except for phytanic and phytenic acid binding to RXRs, where n = 3.

Binding assays were performed as previously described (Allegretto *et al.*, 1993).

probably due to cellular toxicity in which, above 64 μ M, the limits for fatty-acid binding to serum albumin were exceeded (Herndon *et al.*, 1969; Spector *et al.*, 1969). Alternatively, some of the natural isomers of phytanic acid (Baxter and Milne, 1969) may inhibit RXR binding. Integration of the effector activities produced by each of these isomers in the tested sample of phytanic acid may thus give rise to the nonsaturable activity profile. Nevertheless, given that their plasma levels approximate their RXR binding affinities and activation potencies, phytanic acid and phytenic acid remain compelling candidates for humoral RXR effectors.

The units of RXR effector activity caused by phytanic acid were only crudely assessed in our experiments, but the activity caused by the injected serum sample (Figure 2A) can be accounted for by the peak of activity found in fractions 23 and 24. The contribution of phytanic acid to the estimated on the basis of it (0.5 ml) in bovine plasma (Av 10 ml) of the 10 ml-extract assayed for RXR effector activity using the CAT reporter plasmid (Figure 2A). Thus, the estimated phytanic acid (0.025 mg or 80 nmol) in fractions 23 and 24 (Figure 2A) in 4 ml of media is 20 μ M, which approximates the EC₅₀ value in the dose-response curve. Importantly, the induction in this experiment was submaximal, evidence for which is given by the threefold increase (Figure 2A) as compared with the 16-fold maximum induction seen in Figures 1 and 4B. Because phytanic acid and phytenic acid constitute the only RXR-inducing molecular species in serum (Figure 5), both seem to define the bulk of activity.

Distinct Humoral Diterpenoid Activators for RAR and RXR

Circulating ATRA levels are 6 nM (Napoli *et al.*, 1985; Tang and Russel, 1990), which are sufficient for RAR

Our data do not exclude the possibility that 9cRA may be an intracellular signal. Support for 9cRA as a physiological RXR effector comes from a cochromato-

graphing 350-nm absorbance peak detected in mouse liver and kidney extracts (Heyman *et al.*, 1992). Although 9cRA binds and activates RXR with high affinity and potency (Heyman *et al.*, 1992; Levin *et al.*, 1992), the fractions corresponding to this absorbance peak were not tested for RXR activation. Endogenous levels of 9cRA were estimated as 4 ng/g (13 nM) of liver and 30 ng/g (100 nM) of kidney by measuring the areas of absorbance peaks (Heyman *et al.*, 1992). By using the estimate for 9cRA in bovine serum (0.5 nM) calculated above, humoral 9cRA must be 25 times lower than that found in liver. If ATRA serves as the precursor for 9cRA, this cellular metabolite must accumulate against a considerable concentration gradient. Nevertheless, a cellular enzyme may catalyze this isomerization, and, thus, both humoral phytol metabolites and 9cRA synthesized in cells may cooperate to mediate physiological effects through RXR. Molecular structure determination of the RXR effectors extracted from other animal tissues may help to resolve this issue.

Candidate Essential Fatty Acids

Chlorophyll is best recognized as an energy transducer in plants that captures sunlight for oxygen, sugar, and lipid synthesis and thereby establishes the foundation for animal food chains. Phytol metabolites may now strengthen this link between heterotrophs and autotrophs by integrating the dietary state of the animal with RXR-dependent signaling systems to balance the lipid stores in adipose tissue against cellular needs. Insights into the functions of phytol metabolites may emerge from comparisons with linoleic acid and other unsaturated fatty acids that are important dietary factors synthesized by plants (Burr and Burr, 1929, 1930; Aaes-Jorgensen, 1961).

Although they serve as important precursors for prostaglandin synthesis (Bergstrom, 1966), unsaturated fatty acids may also share equally crucial roles as receptor signals. For example, linoleic and arachidonic acids activate PPAR α with a potency of 30 μ M (Gottlicher *et al.*, 1992; Banner *et al.*, 1993; Keller *et al.*, 1993). Linoleic acid may contribute as much as 20% (40 μ M) of the total fatty acids (200 μ M) in human or rat sera (Swell *et al.*, 1961; Scully *et al.*, 1980). Although these levels are within PPAR α activation range, other ligands have been noted for PPAR γ , such as prostaglandin J₂ (Forman *et al.*, 1995b; Kliewer *et al.*, 1995). The distinct pharmacological characteristics noted for different PPARs (Kliewer *et al.*, 1994) may be specified by unique subsets of dietary or endogenously synthesized fatty acids and their oxidized and cyclized metabolites. A strategy similar to that outlined here could also guide the isolation of PPAR effectors from tissue extracts.

Phytanic acid is obtained only from dietary sources and is rapidly oxidized just like other fatty acids, but its specific nutritional requirement is unknown. Abundant sources of phytanic acid in human diets are milk, cheese, and especially butter (Lough, 1977). The caloric value of phytanic acid is only fractionally that of linoleic acid because of their abundance differences, and thus its contribution to cellular energy reserves must be low. It remains uncertain whether pathological states will develop in animals fed phytanic acid-deficient diets. This might be expected, given the number of signaling pathways converging with RXR. Potential pathological signs could overlap those produced by deficiencies of linoleic acid, thyroid hormones, vitamins A and D, or other ligands whose receptors cooperate with RXR. Preparation of diets lacking phytol and its metabolites will be critical for these nutritional studies. Like the fat-soluble vitamins A and E, it may also be necessary to maintain animals on phytanic acid-deficient diets for prolonged times to deplete stored forms of the fatty acid.

It may be of interest to note that linoleic acid deficiency retards animal growth and that butter efficiently restores the weight lost in rats given fat-free diets (Burr and Burr, 1930; Aaes-Jorgensen, 1961). Although linoleic acid has been shown to be one active component, phytanic acid may represent another of their postulated "vitamine F" growth-promoting substances (Evans and Burr, 1928). Phytanic acid could also serve as a growth factor for cells in culture, because linoleic acid replacement of serum albumin and its bound fatty acids has been shown to increase their plating efficiency in serum-free media (Ham, 1963).

Dietary Lipids as Nutritional Signals

Phytol metabolites, unsaturated fatty acids, retinol, and farnesoids may form a unique class of micromolar cellular metabolites that also serve as signals for RXR and some of its receptor partners. Linoleic acid and other unsaturated fatty acids are candidate physiological PPAR effectors that regulate the genes for enzymes involved in lipid metabolism (Keller and Wahli, 1993). The carotenoid metabolite retinol may be an important RAR signal, as discussed above. Similarly, metabolites of farnesyl pyrophosphate (FPP) have been postulated to regulate isoprenoid synthesis through FXR interactions (Forman *et al.*, 1995a; Weinberger, 1996). FPP in liver (0.4 μ M) (Bruenger and Rilling, 1988) is slightly lower than the micromolar farnesoids needed for FXR induction, but activation of a farnesoid metabolic shunt could increase the pool of FXR effectors in some dietary states. Critical evaluation of this hypothesis will require measurements of cellular farnesoid levels and their FXR-binding potentials. Finally, phytol metabolites may be nutritional signals linking the animal's dietary state with a variety

of endocrine and intracrine signaling pathways through the nuclear receptor RXR. As a model, we propose that these dietary lipids may coordinate gene expression for fatty-acid biosynthetic and oxidative enzymes by PPAR and RXR interactions. In conjunction with farnesoids and FXR, these networks could regulate the flux of acetyl CoA from intermediary metabolism to meet cellular lipid needs during variable dietary conditions.

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tabolites in various tissues during different nutritional states could be compared with their RXR-binding properties to further test this hypothesis.

To more firmly establish their physiological relevance, these and other orphan receptor ligands must also comply with a broader set of postulates analogous to those identifying the etiologic roles of bacteria for animal diseases. Physiological effects mediated by phytanic acid must first be described. Animals raised on phytanic acid-depleted diets may offer one way to identify these cell functions specifically controlled by RXR. These assayable end points might then lead to the purification of phytol metabolites from tissue extracts, just as uterine cell changes in ovariectomized mice guided the isolation of estrogenic substances (Allen and Doisy, 1923).

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REFERENCES

- Aaes-Jorgensen, E. (1961). Essential fatty acids. *Physiol. Rev.* 41, 1-41.
- Allen, E., and Doisy, E.A. (1923). An ovarian hormone. Preliminary report on its localization, extraction and partial purification, and action in test animals. *J. Am. Med. Assoc.* 81, 819-821.
- Allegretto, E.A., McClurg, M.R., Lazarchik, S.B., Clemm, D.L., Kerner, S.A., Elgort, M.G., Boehm, M.F., White, S.K., Pike, J.W., and Heyman, R.A. (1993). Transactivation properties of retinoic acid and retinoid X receptors in mammalian cells and yeast. *J. Biol. Chem.* 268, 26625-26633.
- Allenby, G., Bocquel, M.-T., Saunders, M., Kazmer, S., Speck, J., Rosenberger, M., Lovey, A., Kastner, P., Grippo, J.F., Chambon, P., and Levin, A.A. (1993). Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. *Proc. Natl. Acad. Sci. USA* 90, 30-34.
- Arriza, J.L., Weinberger, C., Cerelli, G., Glaser, T.M., Handelin, B.L., Housman, D.E., and Evans, R.M. (1987). Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* 237, 268-275.
- Avignan, J. (1966). The presence of phytanic acid in normal human and animal plasma. *Biochim. Biophys. Acta* 166, 391-394.
- Banner, C.D., Gottlicher, M., Widmark, E., Sjovall, J., Rafter, J.J., and Gustafsson, J.-A. (1993). A systematic analytical chemistry/cell approach to isolate activators of orphan nuclear receptors from biological extracts: characterization of peroxisome proliferator-activated receptor activators in plasma. *J. Lipid Res.* 34, 1583-1591.
- Baxter, J.H., and Milne, G.W.A. (1969). Phytanic acid: identification of five isomers in chemical and biological products of phytol. *Biochim. Biophys. Acta* 176, 265-277.
- Berger, T.S., Parandoosh, Z., Perry, B.W., and Stein, R.B. (1992). Interactions of glucocorticoid analogues with the glucocorticoid receptor. *J. Steroid Biochem. Mol. Biol.* 41, 733-738.
- Bergstrom, S. (1966). The prostaglandins. *Recent Prog. Horm. Res.* 22, 153-175.
- Billimoria, J.D., Clemens, M.E., Gibberd, F.B., and Whitelaw, M.N. (1982). Metabolism of phytanic acid in Refsum's disease. *Lancet* 1(8265), 194-196.
- Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911-917.
- Boehm, M.F., McClurg, M.R., Pathirana, C., Manglesdorf, D., White, S.K., Hebert, J., Winn, D., Goldman, M.E., and Heyman, R. (1994). Synthesis of high specific activity [^3H]-9-*cis*-retinoic acid and its application for identifying retinoids with unusual binding properties. *J. Med. Chem.* 37, 408-414.
- Boehm, M.F., Zhang, L., Zhi, L., McClurg, M.R., Berger, E., Wagoner, M., Mais, D.E., Suto, C.M., Davies, P.J.A., Heyman, R.A., and Nadzan, A.M. (1995). Design and synthesis of potent retinoid X receptor-selective ligands that induce apoptosis in leukemia cells. *J. Med. Chem.* 38, 3146-3155.
- Bruenger, E., and Rilling, H.C. (1988). Determination of isopentenyl diphosphate and farnesyl diphosphate in tissue samples with a comment on secondary regulation of polyisoprenoid biosynthesis. *Anal. Biochem.* 173, 321-327.
- Buck, J., Derguini, F., Levi, K., Nakanishi, K., and Hammerling, U. (1991). Intracellular signaling by 14-hydroxy-4,14-*retro*-retinol. *Science* 254, 1654-1656.
- Burr, G.O., and Burr, M.M. (1929). A new deficiency disease produced by the rigid exclusion of fat from the diet. *J. Biol. Chem.* 82, 345-367.

- Burr, G.O., and Burr, M.M. (1930). On the nature and role of the fatty acids essential in nutrition. *J. Biol. Chem.* 86, 587–621.
- Chen, C., and Okayama, H. (1988). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7, 2745–2752.
- Cheng, Y.C., and Prusoff, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor, which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* 22, 3099–3108.
- Corey, E.J., Gilman, N.H., and Ganem, B.E. (1968). New methods for the oxidation of aldehydes to carboxylic acids and esters. *J. Am. Chem. Soc.* 90, 5616–5617.
- Derguini, F., Nakanishi, K., Hammerling, U., Chua, R., Eppinger, T., Levi, E., and Buck, J. (1995). 13,14-dihydroxyretinol, a new bioactive retinol metabolite. *J. Biol. Chem.* 270, 18875–18880.
- Evans, H.M., and Bishop, K.S. (1923). Existence of a hitherto unknown dietary factor essential for reproduction. *J. Am. Med. Assoc.* 81, 889–892.
- Evans, H.M., and Burr, K.S. (1928). A new dietary deficiency with highly purified diets. III. The beneficial effect of fat in the diet. *Proc. Soc. Exp. Biol. Med.* 25, 390–397.
- Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* 240, 889–895.
- Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., Perlmann, T., Noonan, D.J., Burka, L.T., McMorris, T., Lamph, W.W., Evans, R.M., and Weinberger, C. (1995a). Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* 81, 687–695.
- Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M., and Evans, R.M. (1995b). 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 is a ligand for the adipocyte determination factor PPAR γ . *Cell* 83, 803–812.
- Forman, B.M., Umesono, K., Chen, J., and Evans, R.M. (1995c). Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell* 81, 541–550.
- Giguere, V., Hollenberg, S.M., and Evans, R.M. (1986). Functional domains of the human glucocorticoid receptor. *Cell* 46, 645–652.
- Giguere, V., Ong, E.S., Segui, P., and Evans, R.M. (1987). Identification of a receptor for the morphogen retinoic acid. *Nature* 330, 624–629.
- Glass, C.K. (1994). Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocrinol. Rev.* 15, 391–407.
- Gottlicher, M., Widmark, E., Li, Q., and Gustafsson, J.-A. (1992). Fatty acids activate a chimera of the clofibrate acid-activated receptor and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. USA* 89, 4653–4667.
- Green, S., and Chambon, P. (1987). Oestradiol induction of a glucocorticoid-responsive gene by a chimaeric receptor. *Nature* 324, 615–617.
- Ham, R.G. (1963). Albumin replacement by fatty acids in clonal growth of mammalian cells. *Science* 140, 802–803.
- Harmon, M.A., Boehm, M.F., Heyman, R.A., and Manglesdorf, D.J. (1995). Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. *Proc. Natl. Acad. Sci. USA* 92, 6157–6160.
- Herbomel, P., Bourachot, B., and Yaniv, M. (1984). Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell* 39, 653–662.
- Herndon, J.H., Steinberg, D., Uhlendorf, B.W., and Fales, H.M. (1969). Refsum's disease: characterization of the enzyme defect in cell culture. *J. Clin. Invest.* 48, 1017–1040.
- Heyman, R.A., Manglesdorf, D.J., Dyck, J.A., Stein, R.B., Eichele, G., Evans, R.M., and Thaller, C. (1992). 9-*cis* retinoic acid is a high-affinity ligand for the retinoid X receptor. *Cell* 68, 397–406.
- Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., and Wahli, W. (1993). Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc. Natl. Acad. Sci. USA* 90, 2160–2164.
- Keller, H., and Wahli, W. (1993). Peroxisome proliferator-activated receptors: a link between endocrinology and nutrition? *Trends Endocrinol. Metab.* 4, 291–296.
- Kliwer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Manglesdorf, D.J., Umesono, K., and Evans, R.M. (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. USA* 91, 7355–7359.
- Kliwer, S.A., Lenhard, J.M., Willson, T.M., Patel, I., Morris, D.C., and Lehmann, J.M. (1995). A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell* 83, 813–819.
- Kliwer, S.A., Umesono, K., Heyman, R.A., Manglesdorf, D.J., Dyck, J.A., and Evans, R.M. (1992). Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. *Proc. Natl. Acad. Sci. USA* 89, 1448–1452.
- Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., and Hogness, D.S. (1991). The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67, 59–77.
- Kojima, R., Fujimori, T., Kiyota, N., Toriya, Y., Fukuda, T., Ohashi, T., Sato, T., Yoshizawa, Y., Takeyama, K., Mano, H., Masushige, S., and Kato, S. (1994). In vivo isomerization of retinoic acids: rapid isomer exchange and gene expression. *J. Biol. Chem.* 269, 32700–32707.
- Lehmann, J.M., Jong, L., Fanjul, A., Cameron, J.F., Lu, X.P., Haefner, P., Dawson, M.L., and Pfahl, M. (1992). Retinoids selective for retinoid X receptor-response pathways. *Science* 258, 1944–1946.
- Levin, A.A., Sturzenbecker, L.J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., and Lovey, A. (1992). 9-*cis* retinoic acid stereoisomer binds and activates the nuclear receptor RXR α . *Nature* 355, 359–361.
- Lough, A.K. (1964). Blood lipids. 4. The isolation of 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid) from ox-plasma lipids. *Biochem. J.* 91, 584–588.
- Lough, A.K. (1977). The phytanic acid content of the lipids of bovine tissues and milk. *Lipids* 12, 115–119.
- Manglesdorf, D.J., Borgmeyer, U., Heyman, R.A., Zhou, J.Y., Ong, E.S., Kakizuka, A., and Evans, R.M. (1992). Characterization of three RXR genes that mediate the action of 9-*cis* retinoic acid. *Genes Dev.* 6, 329–344.
- Manglesdorf, D.J., and Evans, R.M. (1995). The RXR heterodimers and orphan receptors. *Cell* 83, 841–850.
- Manglesdorf, D.J., Ong, E.S., Dyck, J.A., and Evans, R.M. (1990). Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 345, 224–229.
- Manglesdorf, D.J., Umesono, K., Kliwer, S.A., Borgmeyer, U., Ong, E.S., and Evans, R.M. (1991). A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell* 66, 555–561.
- Miller, K.W., Lorr, N.A., and Yang, C.S. (1984). Simultaneous determination of plasma retinol, α -tocopherol, lycopene, α -carotene, and

- β -carotene by high-performance liquid chromatography. *Anal. Biochem.* 138, 340–345.
- Mize, C.E., Avignan, J., Baxter, J.H., Fales, H.M., and Steinberg, D. (1966). Metabolism of phytol- $U-^{14}C$ and phytanic acid- $U-^{14}C$ in the rat. *J. Lipid Res.* 7, 692–697.
- Mize, C.E., Herndon, J.H., Jr., Blass, J.P., Milne, G.W.A., Follansbee, C., Laudat, P., and Steinberg, D. (1969). Localization of the oxidative defect in phytanic acid degradation in patients with Refsum's disease. *J. Clin. Invest.* 48, 1033–1040.
- Mohrhauer, H., and Holman, R.T. (1963). The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver. *J. Lipid Res.* 4, 151–159.
- Napoli, J.L., Pramanik, B.C., Williams, J.B., Dawson, M.I., and Hobbs, P.D. (1985). Quantitation of retinoic acid by gas-liquid chromatography-mass spectrometry: total versus all-*trans*-retinoic acid in human plasma. *J. Lipid Res.* 26, 387–392.
- O'Malley, B.W. (1989). Did eucaryotic steroid receptors evolve from intracrine gene regulators? *Endocrinology* 125, 1119–1120.
- O'Malley, B.W. (1990). Orphan receptors: in search of a unifying hypothesis of activation. *Mol. Endocrinol.* 6, 1359–1361.
- Ong, D.E. (1987). Cellular retinol-binding proteins. *Arch. Dermatol.* 123, 1693a–1695a.
- Perlmann, T., and Jansson, L. (1995). A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. *Genes Dev.* 9, 769–782.
- Petkovich, M., Brand, N.J., Krust, A., and Chambon, P. (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 330, 444–450.
- Pijnappel, W.W.M., Hendricks, H.F.J., Folkers, G.E., van den Brink, C.E., Dekker, E.J., Edelenbosch, E., van der Saag, P.T., and Durston, A.J. (1993). The retinoid ligand 4-oxo-retinoic acid is a highly active modulator of positional specification. *Nature* 366, 340–344.
- Repa, J.J., Hanson, K.K., and Clagett-Dame, M. (1993). All-*trans*-retinol is a ligand for the retinoic acid receptors. *Proc. Natl. Acad. Sci. USA* 90, 7293–7297.
- Samuels, H.H., Stanley, F., and Casanova, J. (1979). Depletion of L-3,5,3'-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. *Endocrinology* 105, 80–85.
- Scully, R.E., McNeely, B.U., and Galdabini, J.J. (1980). Normal reference laboratory values. *N. Engl. J. Med.* 302, 37–48.
- Sladek, F.M., Zhong, W., Lai, E., and Darnell, Jr., J.E. (1990). Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.* 4, 2353–2365.
- Song, C., Kokontis, J.M., Hiipakka, R.A., and Liao, S. (1994). Ubiquitous receptor: a receptor that modulates gene activation by retinoic acid and thyroid hormone receptors. *Proc. Natl. Acad. Sci. USA* 91, 10809–10813.
- Sonneveld, W., Haverkamp Begemann, P., van Beers, G.J., Keuning, R., and Schogt, J.C.M. (1962). 3,7,11,15-tetramethylhexadecanoic acid, a constituent of butterfat. *J. Lipid Res.* 3, 351–355.
- Spector, A.A., John, K., and Fletcher, J.E. (1969). Binding of long-chain fatty acids to bovine serum albumin. *J. Lipid Res.* 10, 56–67.
- Steinberg, D. (1983). Phytanic acid storage disease (Refsum's disease). In: *The Metabolic Basis of Inherited Disease*, ed. J.B. Stanbury, J.B. Wyngaarden, D.S. Frederickson, J.L. Goldstein, and M.S. Brown, New York: McGraw-Hill, 731–747.
- Steinberg, D., Avignan, J., Mize, C. (1965). Conversion of $U-^{14}C$ -phytol to phytanic acid and its oxidation in *heredopathia atactica polyneuritiformis*. *Biochem. Biophys. Res. Commun.* 19, 783–789.
- Steinberg, D., Avignan, J., Mize, C.E., Baxter, J.H., Cammermeyer, J., Fales, H.M., and Highet, P.F. (1966). Effects of dietary phytol and phytanic acid in animals. *J. Lipid Res.* 7, 684–691.
- Steinberg, D., Mize, C.E., Avignan, J., Fales, H.M., Eldjarn, L., Try, K., Stokke, O., and Refsum, S. (1967). Studies on the metabolic error in Refsum's disease. *J. Clin. Invest.* 46, 313–322.
- Sucov, H.M., Murakami, K.H., and Evans, R.M. (1990). Characterization of an autoregulated response element in the mouse retinoic acid receptor-type beta gene. *Proc. Natl. Acad. Sci. USA* 87, 5392–5396.
- Swell, L., Law, M.D., Schools, P.E., Jr., and Treadwell, C.R. (1961). Tissue lipid fatty acid composition in pyridoxine-deficient rats. *J. Nutr.* 74, 148–156.
- Tang, G., and Russell, R.M. (1990). 13-*cis* retinoic acid is an endogenous compound in human serum. *J. Lipid Res.* 31, 175–182.
- Teboul, M., Enmark, E., Li, Q., Wikstrom, A.C., Pelto-Huikko, M., and Gustafsson, J.-A. (1995). OR-1, a member of the nuclear receptor superfamily that interacts with the 9-*cis*-retinoic acid receptor. *Proc. Natl. Acad. Sci. USA* 92, 2096–2100.
- Umesono, K., Murakami, K., Thompson, C.C., and Evans, R.M. (1991). Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D₃ receptors. *Cell* 65, 1255–1266.
- Wang, L.-H., Tsai, S.Y., Cook, R.G., Beattie, W.G., Tsai, M.-J., and O'Malley, B.W. (1989). COUP transcription factor is a member of the steroid receptor superfamily. *Nature* 340, 163–166.
- Weinberger, C. (1996). A model for farnesoid feedback control in the mevalonate pathway. *Trends Endocrinol. Metab.* 7, 1–6.
- Willy, P.J., Umesono, K., Ong, E.S., Evans, R.M., Heyman, R.A., and Manglesdorf, D.J. (1995). LXR, a nuclear receptor that defines a distinct retinoid-responsive pathway. *Genes Dev.* 9, 1033–1045.
- Yao, T.-P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., McKewon, M., Cherbas, P., and Evans, R.M. (1993). Functional ecdysone receptor is the product of the *EcR* and *ultraspiracle* genes. *Nature* 366, 476–479.
- Yamamoto, K.R. (1985). Steroid receptor-regulated transcription of specific genes and gene networks. *Annu. Rev. Genet.* 19, 209–252.